APPENDIX 3

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The Fibrinogen Binding Protein of *Staphylococcus epidermidis* Is a Target for Opsonic Antibodies

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Antibodies against the fibrinogen binding protein (Fbe) of *Staphylococcus epidermidis* significantly increased macrophage phagocytosis. Antibodies against autolysin E were opsonic but to a lesser extent. Antibodies against a novel, putatively surface-located antigen were unable to enhance phagocytosis. The severity of systemic infection of mice with *S. epidermidis* was reduced if the bacteria were preopsonized with anti-Fbe prior to administration. Fbe is thus a strong candidate for protein vaccination against *S. epidermidis* infection, and antibodies against Fbe can be used to prevent or treat infections caused by *S. epidermidis*.

Staphylococcus epidermidis is a major nosocomial pathogen. It is a common etiologic agent in neonatal septicemia and in peritonitis, causing mainly foreign body-associated infections. In patients, proteins such as albumin, fibrinogen, fibronectin, and von Willebrand factor rapidly cover implants (3). *S. epidermidis* possesses an unknown number of proteins that contribute to specific adhesion to these molecules. Several studies also demonstrate the important roles of biofilm formation and the polysaccharide intracellular adhesin/hemagglutinin in pathogenicity (17, 18).

Fbe is a 119-kDa fibrinogen binding protein located at the surface of *S. epidermidis* and is present in most tested strains of *S. epidermidis* (11). Fbe has also been named SdrG (1). It is structurally related to other staphylococcal surface-located proteins, such as ClfA of *S. aureus* (9, 15). *S. epidermidis* interaction with peripheral venous catheters from patients, which spontaneously become coated with fibrinogen (5), is blocked by anti-Fbe antibodies (13). Fbe is expressed on the surface of *S. epidermidis* during infection, as indicated by an increase of anti-Fbe titers in sera from patients infected with *S. epidermidis* (8).

Macrophages are of vital importance in suppressing *S. epidermidis* infections (10). Phagocytic activity of macrophages is greatly enhanced if specific antibodies are attached to the pathogen (12).

In this study, we demonstrate that fresh alveolar macrophages from rat ingest and kill *S. epidermidis* opsonized with anti-Fbe antibodies to a much higher extent than they ingest and kill nonopsonized bacteria or bacteria opsonized with antibodies directed against AtlE (4) or another putative surface antigen, Embp (19).

In the first experiment, *S. epidermidis* (strain 19) was opsonized with antibodies and added to macrophages. The bacteria, grown in broth for 2 h (optimal for expression of fibrinogen binding), were washed, adjusted to a concentration of 10^8 CFU/ml, mixed with antibodies (300-μg/ml final concentration), and incubated at 37°C for 1 h. The antibodies used for opsonization were rat or rabbit antibodies against three antigens: glutathione-*S*-transferase fused to Fbe (GST-Fbe), Embp, and AtlE. As controls, phosphate-buffered saline or antibodies taken before immunization were used. GST-Fbe was produced as previously described (15). Embp is a fibronectin binding protein (19). The fibronectin binding domain of Embp, a 207-amino-acid region, was amplified by PCR using DNA from *S. epidermidis* strain 19 with primers 5′-AATTAACCATGGCTGATAAGAATTTACAAATTGAAT (forward) and 5′-AATTAACCCGGGTATTGCAGCTTTTTGAGCATT (reverse); extending NcoI and SmaI sites, respectively, are underlined. Pwo DNA polymerase was used. Running cycles were as follows: 2 min at 96°C followed by 30 cycles of 15 s at 96°C, 30 s at 50°C, and 2 min (prolonged by an additional 20 s for each cycle after the 11th cycle) at 72°C. Cleavage of the PCR product with NcoI and SmaI permitted directional in-frame cloning into the expression vector pTYB4 belonging to the IMPACT T7 system (New England Biolabs). The ligated DNA was used to electrotransform *Escherichia coli* strain ER2566. A plasmid harboring the insert was isolated from a transformant and verified by DNA sequencing. The resulting protein is hereafter termed Embp, although it contains only the fibronectin binding portion. Expression and purification of Embp was performed as described in the protocol provided from the manufacturer of the IMPACT T7 system.

Autolysin E, AtlE, is a 60-kDa surface-located homologue of *S. aureus* major autolysin. It mediates adherence to polystyrene materials and binds specifically to vitronectin (4, 7). An *S. epidermidis* mutant lacking AtlE is less infectious in a rat central venous catheter infection model, indicating a role for AltE in pathogenesis (16). AtlE was a kind gift from Friedrich Götz, Mikrobielle Genetik, Universität Tübingen, Tübingen, Germany.

Antibodies against these antigens were raised in rabbit, rat, or sheep. Subcutaneous immunizations were done at 2-week intervals by using 20 μg of protein. Freund's complete adjuvant was used at the first immunization, which was followed by two booster doses of Freund's incomplete adjuvant. One rat was mock immunized with Freund's adjuvant alone. Antibodies were purified from immune and preimmune sera by separation

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TABLE 1. Phagocytosis of *S. epidermidis* strain 19 opsonized with different antibodies[a]

Origin of antibody	Antibody	Mean $\log_{10}$ no. of CFU ingested	SD in $\log_{10}$ no. of CFU ingested
Rat	Fbe	5.5	4.9
	Embp	3.3	2.4
	Normal IgG	3.2	3.0
Rabbit	Fbe	5.7	5.3
	AtlE	4.8	4.6
	Normal IgG	3.8	3.7
Sheep	Fbe	5.6	5.5
	Normal IgG	3.8	3.7

[a] $\log_{10}$ numbers of CFU of ingested bacteria are shown. *S. epidermidis* strain 19 (10^7 CFU), cultured for 2 h, was opsonized with purified antibodies (300 μg/ml) against Fbe, Embp, AtlE, or normal IgG. The antibodies originated from rat, rabbit, or sheep. Significantly (*t* test) more bacteria opsonized with anti-Fbe antibodies were ingested by alveolar macrophages isolated by rat BAL than bacteria opsonized with anti-AtlE ($P < 0.001$), anti-Embp ($P < 0.001$), or normal IgG ($P < 0.001$). Bacteria opsonized with anti-AtlE were ingested to a higher extent than bacteria opsonized with normal IgG ($P < 0.05$). Standard deviations are shown ($n = 4$ for all tests).

on protein G Sepharose 4 fast flow (Pharmacia Amersham). Therefore, all phagocytosis experiments were performed without complement present. The antibody concentration was set to ca. 3 mg/ml. Total and specific immunoglobulin G (IgG) titers were determined for each antibody or serum preparation by conventional enzyme-linked immunosorbent assay (data not shown). The coating concentrations were 1 μg/well for GST-Fbe and AtlE and 0.1 μg/well for Embp. No cross-reactivity among the proteins was found.

Rabbit sera against GST-Fbe or AtlE were gifts from Åsa Ljungh, Lund University. Sheep sera against GST-Fbe were gifts from Per Månsson, SBL Vaccine, Stockholm, Sweden.

For macrophage ingestion studies, 10^7 opsonized bacteria in culture media were added to the macrophages attached to 24-well plates. Macrophages were obtained from rats (average weight, 180 g; Wistar) by bronchoalveolar lavage as described elsewhere (2, 6). The macrophages were adjusted to a concentration of 10^6 cells/ml and added to a 24-well cell culture plate (Nunc) at 10^6 cells/well. The plates were stored at 4°C for 1 h for adherence, and then red blood cells and other nonadherent cells were washed away with Hanks balanced salt solution (Gibco). The plates with infected macrophages were incubated with 7% CO_2 at 37°C. The optimal incubation time was found to be 2 h. Extracellular bacteria were gently washed away, and the remaining bacteria were killed by treatment with lysostaphin at 50 μg/ml for 30 min. The plates were centrifuged at 110 × *g*, and the supernatants were discarded. The cells were lysed and detached with 1 M NaCl. Microscopic examination revealed lysis of all eukaryotic cells. The bottoms of the wells were scraped, and the contents were serially diluted and plated onto blood agar plates.

As shown in Table 1, the number of bacteria ingested into macrophages varied considerably depending on the opsonizing antibody used. Maximum ingestion was equivalent to 10 bacteria/macrophage, corresponding to half the number of the inoculated bacteria. With no antibodies added, less than 0.01 CFU/cell was found after 2 h of incubation.

Of the antibodies tested, only antibodies against Fbe and AtlE were effective as opsonizers. *S. epidermidis* strain 19 was ingested 177 times better when opsonized with rat anti-Fbe antibodies than when opsonized with the rat preimmune antibody controls ($P < 0.001$) and 144 times better than when opsonized with rat antibodies against Embp ($P < 0.001$). Antibodies directed against Embp were no better opsonizers than phosphate-buffered saline (data not shown) or preimmune antibodies ($P > 0.05$). Rabbit anti-AtlE antibodies enhanced phagocytosis by 11 times ($P < 0.05$) compared with normal rabbit IgG controls. However, rabbit anti-Fbe antibodies were eight times more effective in opsonization than rabbit anti-AtlE ($P < 0.001$) and 80 times more effective than normal IgG ($P < 0.001$). Sheep antibodies directed against Fbe enhanced phagocytosis by 60 times ($P < 0.001$) compared with preimmune IgG from the same animal (Table 1).

Quantification was also done by using serial dilutions of rabbit antibodies. An approximately 20-fold-higher concentration of anti-AtlE antibodies was required to reach the same level of phagocytosis of *S. epidermidis* strain 19 as that reached with anti-Fbe antibodies (data not shown).

To study the rate of killing of ingested bacteria, macrophages which had ingested bacteria for 2 h were kept for an additional 0, 2, and 16 h after treatment with lysostaphin. Intra- and extracellular bacteria were then enumerated. The bacteria could persist intracellularly for more than 2 h after termination of phagocytosis and even seemed to increase in numbers. However, after 18 h of incubation there was substantial killing; only about 1% of the ingested bacteria remained after this time (data not shown).

The bacterial strain variation was assessed by using seven different strains of *S. epidermidis* and one *S. aureus* strain in opsonization experiments with anti-Fbe antibodies. All the strains possess the *fbe* gene, except STO 56 (*fbe::eryR* mutant isogenic with strain HB) (14) and *S. aureus* strain Newman. All strains tested were better phagocytosed after opsonization with anti-Fbe antibodies than after opsonization with preimmune antibodies. The enhancement factors were 108 (for strain 19), 93, 45, 15, 1.5, and 1.2 ($P < 0.05$ for all strains). The strains lacking Fbe, *S. epidermidis* STO 56 and *S. aureus* Newman, were not stimulated at all with anti-Fbe antibodies (data not shown).

All these strains were also tested for their capacity to bind to fibrinogen by using a method described earlier (11). Although all strains have the *fbe* gene, binding to fibrinogen varied considerably (data not shown), presumably due to various levels of expression of Fbe or various levels of exposure on the surface.

A correlation between the fibrinogen binding capacity of these strains and opsonization with anti-Fbe antibodies was clearly seen. The correlation between the two parameters is linear with a correlation coefficient *r* of 0.83 ($P < 0.05$) as determined using the least-square method. Thus, opsonization with antibodies against Fbe increases phagocytosis and killing of *S. epidermidis* strains relative to their fibrinogen binding capacities.

To evaluate the protective effect of anti-Fbe antibodies in vivo, opsonized bacteria were given intravenously to mice. *S. epidermidis* strain 19 was concentrated to 10^9 CFU/ml and incubated with 10% serum for 1 h at 37°C, and 100 μl was given intravenously to ~30-g female Naval Medical Research Institute (NMRI) mice (25 mice/group). The serum used was sheep anti-GST-Fbe (obtained from Per Månsson, SBL Vac-

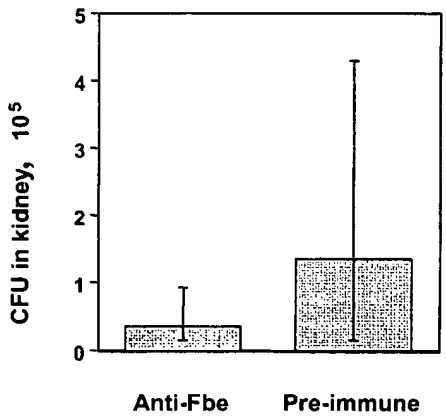

FIG. 1. Bacterial loads in mouse kidneys after infection with preopsonized *S. epidermidis* strain 19. Mice were infected with bacteria preopsonized with serum against Fbe or normal serum. Bacterial loads in kidneys after 4 days were significantly lower ($P < 0.05$; median test) in mice inoculated with bacteria preopsonized with anti-Fbe (median, 3.2×10^4 CFU; upper quartile, 9.1×10^4 CFU; lower quartile, 8.2×10^3 CFU) than in those inoculated with bacteria preopsonized with normal serum (median, 1.3×10^5 CFU; upper quartile, 4.3×10^5 CFU; lower quartile, 5.9×10^3 CFU).

cine) or normal sheep serum as a control. The severity of bacterial infection was determined by measuring the bacterial numbers in the kidneys after 4 days. The bacterial load in the kidneys is an indication of the total bacterial load in the body. As shown in Fig. 1, preopsonization of bacteria with anti-Fbe serum led to a lower level of bacterial proliferation (median, 3.2×10^4 CFU; upper quartile, 9.1×10^4 CFU; lower quartile, 8.2×10^3 CFU) than preopsonization of bacteria with normal serum (median, 1.3×10^5 CFU; upper quartile, 4.3×10^5 CFU; lower quartile, 5.9×10^3 CFU) ($P < 0.05$), thus indicating a protective effect of these antibodies.

Taken together, these findings show that antibodies against Fbe have the capacity to opsonize and increase phagocytosis of *S. epidermidis* in vitro and to decrease the severity of experimental infection in mice. Fbe is thus a strong candidate for continued development of antibody-mediated therapy or prophylaxis against infection.

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